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Clinical Studies

The dynamic change of circulating tumour cells in patients with operable breast cancer before and after chemotherapy based on a multimarker QPCR platform

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BACKGROUND: The possible presence of early tumour dissemination is the rationale behind the use of systemic adjuvant chemotherapy in patients with operable breast cancer. Circulating tumour cells (CTC) in peripheral blood may represent the possible presence of early tumour dissemination. However, relatively few studies were designed to investigate the relationship between the change of CTC status and the efficacy of adjuvant chemotherapy in operable breast cancer patients.

METHODS: In a prospective study, we established a multimarker real-time quantitative PCR platform to detect CTC in peripheral blood of breast cancer patients. By using this platform, we detected CTC in peripheral blood of 94 operable breast cancer patients. Control group consisted of 20 patients with benign breast disease and 20 healthy volunteers. For 72 patients who underwent systemic adjuvant chemotherapy, the dynamic CTC status at three different time points (1 day before initiation of chemotherapy, 1 week after three cycles of chemotherapy and 1 week after all cycles of chemotherapy) was observed.

RESULTS: Circulating tumour cells were detected in 56% (53 out of 94) of patients with operable breast cancer. The specificity was 95%. Seventy-two patients who received systemic adjuvant chemotherapy were followed up. After three cycles of chemotherapy, 47% (18 out of 38) of patients who were CTC-positive before chemotherapy changed into negative status. In addition, another 5% (2 out of 38) of patients had changed into negative status after all cycles of chemotherapy.

CONCLUSION: Systemic adjuvant chemotherapy had a significant impact on CTC status, and this effect could be observed after three cycles of chemotherapy. Circulating tumour cells detection had the potential to be used to evaluate the efficacy of systemic adjuvant chemotherapy immediately after the chemotherapy was finished in operable breast cancer patients.

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Metastasis is the main cause of death in breast cancer patients. Either by haematal routes or by lymphangial routes, breast cancer cells can invade into circulation system and finally cause distant metastasis. Breast cancer cells can be detected in peripheral blood, which are known as circulating tumour cells (CTC). Many studies have confirmed that CTC can be detected in the early stage of breast cancer (Pierga *et al*, 2004; Ring *et al*, 2004; Benoy *et al*, 2006; Pierga *et al*, 2007), and the presence of CTC is associated with haematogenous dissemination and bad prognosis (Wulfing *et al*, 2006; Xenidis *et al*, 2006; Molloy *et al*, 2011).

In clinical practice, the possible presence of hematogenous dissemination is the rationale behind the use of adjuvant chemotherapy in operable breast cancer patients (Schabel, 1977; Pantel and Brakenhoff, 2004). Indeed, the efficacy of adjuvant chemotherapy can be assessed currently only retrospectively in clinical trials, following an observation period of at least 5 years. Consequently, the introduction of new therapies in the clinic takes a long time. Especially, it is not possible to tailor therapy to an individual patient. By contrast, CTC can be detected dynamically by sequential sampling of peripheral blood; hopefully, it can be used to evaluate the efficacy of adjuvant chemotherapy immediately after chemotherapy is finished. However, owing to the heterogeneity of breast cancer cells and instability of gene expression, no single marker can represent all kinds of breast cancer cells (Perou *et al*, 2000), so multimarker assays with both high sensitivity and specificity are required. On the other hand, there are very few CTC present in the blood of operable breast cancer patients, compared with metastatic patients. In this respect, it has been difficult to show a prognostic value of CTC analyses (Benoy *et al*, 2006; Pierga *et al*, 2007). Therefore, relatively few studies were designed to investigate the relationship between the change of CTC status and the efficacy of chemotherapy in operable breast cancer patients.

In the present study, by combining three widely used mRNA markers, including cytokeratin 19 (CK19), human mammaglobin (hMAM) and small breast epithelial mucin (SBEM) (Miksicek *et al*, 2002; Benoy *et al*, 2004; Roncella *et al*, 2008; Daskalaki *et al*, 2009; Tjensvoll *et al*, 2009; Valladares-Ayerbes *et al*, 2009; Van der

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Auwera *et al*, 2010; Molloy *et al*, 2011), we established a multimarker real-time quantitative PCR (QPCR) platform to detect CTC in peripheral blood of breast cancer patients. Then, we sequentially observed the dynamic CTC status in peripheral blood of operable breast cancer patients who underwent systemic adjuvant chemotherapy at three different time points (1 day before initiating of chemotherapy, 1 week after three cycles of chemotherapy and 1 week after all cycles of chemotherapy), and tried to figure out whether CTC detection had the potential to be used to evaluate the efficacy of adjuvant chemotherapy immediately after chemotherapy was finished in operable breast cancer patients.

MATERIALS AND METHODS

Breast cancer cell line

MCF-7 cells were maintained in RPMI 1640 (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO) and 1% penicilin-streptomycin solution (GIBCO). Cells were grown at 37 °C in a 5% CO_2 atmosphere incubator.

Patient selection

Ninety-four patients with operable breast cancer (aged 28–85 years) treated at the Department of Breast Surgery of Jiangsu Province Hospital between 2010 and 2011 were recruited in this study consecutively and were sampled for the first time before surgery. For every patient enroled, a complete diagnostic evaluation was done consisting of chest X-rays, mammography, liver ultrasonography and whole-body bone scan to exclude distant metastasis. For 72 patients who underwent systemic adjuvant chemotherapy (according to NCCN 2010 guidelines of breast cancer), peripheral blood samples were additionally obtained at three different time points (1 day before initiation of chemotherapy, 1 week after three cycles of chemotherapy, 1 week after all cycles of chemotherapy).

In order to define baseline expression in peripheral blood of the multiple mRNA markers used in this study, peripheral blood from control subjects was collected. Twenty healthy volunteers (aged 26–72 years) and 20 patients with benign breast disease (aged 28–65 years) treated at the Department of Breast Surgery of Jiangsu Province Hospital between 2010 and 2011 were enroled as control subjects. None of the control subjects had a history or clinical evidence of malignancy.

All of the patients and donors gave their informed consent, and the study has been approved by the Ethical and Scientific Committee of our Institution.

Blood processing

To reduce blood contamination by epithelial cells from the skin, the first 2 ml of blood was discarded and the collection tube was disconnected before withdrawing the needle at the end of the procedure. Peripheral blood (10 ml in EDTA) was collected and then shipped at room temperature within 2 h to the molecular diagnostic laboratory for immediate processing by Ficoll density gradient centrifugation (Ficoll-Paque Plus; TBDscience, Tianjin, China), and cell pellets were kept at − 80 °C until RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was isolated from blood samples and cell line using TRIzol Reagent (TaKaRa, Dalian, China) according to the manufacturer's protocol. Quantification and purity assessment were performed by optical density measurement at 260 and 280 nm. The RNA quality was also checked by 1.5% non-denaturing agarose gel electrophoresis. RNA integrity was tested by real-time QPCR amplification of the β-actin housekeeping gene. RNA samples from the human breast cancer cell line MCF-7 served as a positive control.

Reverse transcription of RNA was carried out with the PrimeScript RT Master Mix system (TaKaRa). Complimentary DNA was synthesised from 2 μg of total RNA isolated from peripheral blood mononuclear cells of healthy volunteers, breast benign disease patients and breast cancer patients in a total volume of 40 μl, according to the manufacturer's instructions.

Primers

The primers of *CK19*, *SBEM* and the 'housekeeping gene' *β-actin* were designed by the Primer5 software (PREMIER, Palo Alto, CA, USA), and hMAM primer was derived from a previously published article (Benoy *et al*, 2004). All primers were designed to be intron-spanning to preclude amplification of genomic DNA. All primers were synthesised by Invitrogen (Shanghai, China). The forward and reverse primers are listed in Table 1. Serially diluted cDNA synthesised from RNA of human breast cancer cell line MCF-7 was used to generate standard curves of control and marker genes. The efficiency of the primers *β-actin*, *CK19*, *hMAM* and *SBEM* was 1.00, 0.97, 0.98 and 0.96, respectively.

Multimarker real-time QPCR

Real-time QPCR for the 'housekeeping gene' β-actin and marker genes (*CK19*, *hMAM* and *SBEM*) was performed for every cDNA sample either from control subjects or from breast cancer patients. All PCR reactions were performed on the Eppendorf realplex (Eppendorf, Hamburg, Germany) using the fluorescent SYBR Green I methodology. The PCR cycle at which the fluorescence arises above the background signal is called the cycle threshold (Ct).

Real-time QPCR was performed with SYBR Premix Ex Taq (TaKaRa) according to the manufacturer's instructions in a final volume of 20 μl containing 10 μl of 2 × SYBR Premix Ex Taq, 0.4 μl of 10 μM PCR forward primer, 0.4 μl of 10 μM PCR reverse primer, 0.4 μl of 50 × ROX reference dye, 2 μl of cDNA template and 6.8 μl of dH_2O. The thermal cycling conditions comprised 10 min at 95 °C and 40 cycles of 10 s denaturation at 95 °C, 15 s annealing at 55 °C and 45 s extension at 72 °C.

The *CK19*, *hMAM* and *SBEM* mRNA quantities were analysed in triplicate, normalised against β-actin as a control gene. As described by Livak and Schmittgen (2001), results are expressed as relative gene expression (RGE) using the $2^{-\Delta\Delta Ct}$ method. Relative gene expression value '1' of the multiple mRNA markers (*CK19*, *hMAM* and *SBEM*) was derived from the mRNA expression in peripheral blood of a healthy volunteer.

Real-time QPCR positivity was defined as gene expression beyond the cut-off threshold, which was set for each gene marker at three s.d. from the mean expression in healthy control blood samples (Mikhitarian *et al*, 2008). Positivity shown for the

Table 1 Primer sequences

Gene	Sequence (5′–3′)
CK19	Sense: TCCGAACCAAGTTTGAGACG
	Antisense: CCCTCAGCGTACTGATTTCCT
hMAM	Sense: ATGAAGTTGCTGATGGTCCTCAT
	Antisense: GTCTTAGACACTTGTGGATTGATTGTCT
SBEM	Sense: GTATCCAGCTACTGGTCCTGCT
	Antisense: CAATTGCAGAAGACTCAAGCTG
β-actin	Sense: GCTGTGCTATCCCTGTACGC
	Antisense: TGCCTCAGGGCAGCGGAACC

Abbreviations: *β-actin* = beta-cytoplasmic actin 2; *CK19* = cytokeratin 19; *hMAM* = human mammaglobin; *SBEM* = small breast epithelial mucin.

multimarker method is defined as positivity for at least one of the markers.

Statistical analysis

All data were analysed by the Statistical Package for the Social Science version 12.0 (SPSS, Chicago, IL, USA). The Pearson's χ^2 test or, in the case of low frequencies per cell, the Fisher's exact method was used to assess the relationship between rates of positive samples and patient characteristics. The Wilcoxon test for paired non -normally distributed groups was used to compare the dynamic change of CTC status before and after systemic adjuvant chemotherapy. Two-sided P values <0.05 were considered statistically significant.

RESULTS

Study inclusion and patient characteristics

A total of 94 patients with operable breast cancer were included and peripheral blood was obtained for the first time before surgery. Seventy-two patients who underwent systemic adjuvant chemotherapy were additionally sampled 1 day before the initiation of chemotherapy, 1 week after three cycles of chemotherapy and 1 week after all cycles of chemotherapy. The median age was 54.5 years (range: 28–85 years), 79% (74 out of 94) of patients were of >45 years, 60% (56 out of 94) of patients were of postmenopausal status, 28% (26 out of 94) of patients had stage III disease at diagnosis, 70% (66 out of 94) of patients had tumours >2 cm, 48% (45 out of 94) of patients had infiltrated axillary lymph nodes, 29% (27 out of 94) of patients had tumours with histological grade 3, 30% (28 out of 94) of patients had tumours that were ER- and PR-negative and 27% (25 out of 94) of patients had tumours that were HER2-positive (Table 2). Totally, 77% (72 out of 94) of patients underwent systemic adjuvant chemotherapy.

RGE of multiple markers in normal control peripheral blood samples

In this study, baseline gene expression was precisely quantitated in 40 normal peripheral blood samples by real-time QPCR (Table 3). The median RGE value of *CK19*, *hMAM* and *SBEM* in normal control peripheral blood cells was 0.88, 1.38 and 1.04, respectively. To get maximum specificity, a cut-off value for marker positivity, that is, abnormal expression was set at three s.d. from the mean $2^{-\Delta\Delta Ct}$ value for each gene. The cut-off RGE value set up for *CK19*, *hMAM* and *SBEM* was 3.77, 6.62 and 3.27, respectively.

A multimarker real-time QPCR analysis of CTC in patients with operable breast cancer

The median RGE value of *CK19*, *hMAM* and *SBEM* in the peripheral blood of patients with operable breast cancer was 2.51, 3.34 and 1.41, respectively. It was found that 49% (46 out of 94) of patients were positive for *CK19*, 34% (32 out of 94) of patients were positive for *hMAM* and 13% (12 out of 94) of patients were positive for *SBEM* (Table 3). Totally, 56% (53/94) of patients were positive for at least one marker. The sensitivity of the individual marker *CK19*, *hMAM* and *SBEM* was 49%, 34% and 13%, respectively, and the specificity was 97.5%, 97.5% and 100%, respectively. By using the multimarker method, the sensitivity was 56% and the specificity was 95% (Table 4). Neither distribution of patients nor primary tumour tissue characteristics was significantly different between the CTC-positive and CTC-negative patients ($P>0.05$) (Table 2).

Table 2 Clinical characteristics of operable breast cancer patients ($n=94$), according to the CTC status

Patients (*n*)	Total *n* = 94	CTC-positive (within group%) *n* = 53	CTC-negative (within group%) *n* = 41	*P*-value
Age (years)				
⩽45	20 (21)	13 (25)	7 (17)	0.381
>45	74 (79)	40 (75)	34 (83)	
Menopausal status				
Premenopausal	38 (40)	22 (42)	16 (39)	0.808
Postmenopausal	56 (60)	31 (58)	25 (61)	
Tumour size[a]				
T1	28 (30)	14 (26)	14 (34)	0.169
T2	62 (66)	35 (66)	27 (66)	
T3	4 (4)	4 (8)	0 (0)	
Node stage[a]				
N0	49 (52)	25 (47)	24 (59)	0.493
N1	20 (21)	12 (23)	8 (20)	
N2	13 (14)	7 (13)	6 (15)	
N3	12 (13)	9 (17)	3 (7)	
Stage of disease[a]				
I	17 (18)	9 (17)	8 (20)	0.553
II	51 (54)	27 (51)	24 (59)	
III	26 (28)	17 (32)	9 (22)	
Histological grade				
Grade 1	26 (28)	14 (26)	12 (29)	0.204
Grade 2	41 (44)	20 (38)	21 (51)	
Grade 3	27 (29)	19 (36)	8 (20)	
Hormone receptor				
Negative	28 (30)	14 (26)	14 (34)	0.416
ER- and/or PR-positive	66 (70)	39 (74)	27 (66)	
HER2 status[b]				
Negative	69 (73)	36 (68)	33 (80)	0.172
Positive	25 (27)	17 (32)	8 (20)	
Adjuvant chemotherapy				
Yes	72 (77)	38 (72)	34 (83)	0.202
No	22 (23)	15 (28)	7 (17)	

Abbreviations: CTC = circulating tumour cells; CTC-positive or CTC-negative = positive or negative circulating tumour cell status according to the multimarker real-time QPCR platform, QPCR positivity was defined as gene expression beyond the cut-off threshold, which was set for each gene marker at three s.d. from the mean expression in healthy control blood samples, the multimarker platform positivity is defined as positivity for at least one of the markers; ER = oestrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2; QPCR = quantitative PCR. [a]TNM 6 classification according to the Union Internationale Contre le Cancer criteria. [b]HER2 positivity = 3+ in immunohistochemistry or positive fluorescent *in situ* hybridisation test.

The dynamic change of CTC status before and after chemotherapy

Among 72 patients who underwent systemic adjuvant chemotherapy, 53% (38 out of 72) of patients were CTC-positive and 47% (34 out of 72) of patients were CTC-negative before initiation of chemotherapy. Patients who were CTC-negative before chemotherapy continued to be negative after chemotherapy. Among 38 patients with positive status before chemotherapy, 18 patients changed into negative status after three cycles of chemotherapy and another two patients changed into negative status after all cycles of chemotherapy. In total, 53% (20 out of 38) of patients with positive status before chemotherapy changed into negative status after chemotherapy (Figure 1, Table 5). Circulating tumour

cells' status had a significant change before and after chemotherapy, and systemic adjuvant chemotherapy had a significant impact on CTC status in patients with operable breast cancer ($P<0.01$).

DISCUSSION

There are three widely used methods for CTC detection: the CellSearch System (Veridex LLC, Raritan, NJ, USA), the AdnaTest Breast Cancer Select/Detect (AdnaGen AG, Langenhagen, Germany) and multimarker real-time QPCR assays. In a study by Van der Auwera *et al* (2010), the sensitivity of the multimarker (*CK19* and *hMAM*) real-time QPCR assay significantly exceeded that of the CellSearch System (Veridex LLC) and the AdnaTest. In the present study, three widely used markers, including *CK19*, *hMAM* and *SBEM*, were tested by real-time QPCR in the peripheral blood of breast cancer patients. Results showed that *CK19*-, *hMAM*- and *SBEM*-mRNA were positive in 49% (46 out of 94), 34% (32 out of 94) and 13% (12 out of 94) of the operable breast cancer patients, respectively (Table 4). However, when using the multimarker assay (positivity is defined as positivity for at least one of the markers), the sensitivity was improved to 56% (53 out of 94), which was higher than other similar studies (Pierga *et al*, 2008; Daskalaki *et al*, 2009; Xenidis *et al*, 2009; Chen *et al*, 2010; Krishnamurthy *et al*, 2010); meanwhile the specificity was 95% (38 out of 40). It shows the multimarker real-time QPCR assay we used has both high sensitivity and specificity for CTC detection. However, because of lack of prognostic data, our results should be adopted cautiously.

Table 3 RGE of multiple markers in peripheral blood of control subjects and operable breast cancer patients

	Control subjects	Operable breast cancer patients
RGE of CK19		
N	40	94
Median	0.88	2.51
Minimum	0.52	0.42
Maximum	5.89	53.38
Cut-off[a]	3.77	3.77
No. of patient > cut-off	1	46
RGE of hMAM		
N	40	94
Median	1.38	3.34
Minimum	0.72	0.55
Maximum	6.88	509.64
Cut-off[a]	6.62	6.62
No. of patient > cut-off	1	32
RGE of SBEM		
N	40	94
Median	1.04	1.41
Minimum	0.56	0.58
Maximum	2.79	20.44
Cut-off[a]	3.27	3.27
No. of patient > cut-off	0	12

Abbreviations: *CK19* = cytokeratin 19; *hMAM* = human mammaglobin; QPCR = quantitative PCR; RGE = relative gene expression; *SBEM* = small breast epithelial mucin. [a]The cut-off was set for each gene marker at three s.d. from the mean expression in healthy control blood samples.

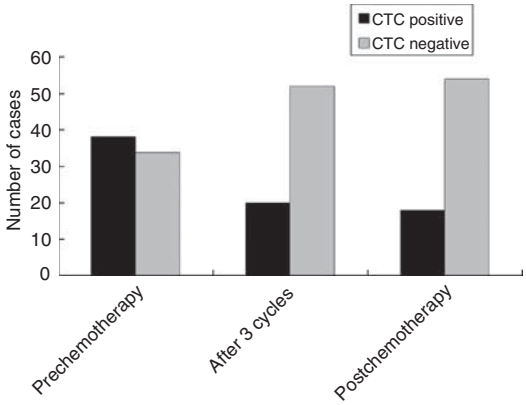

Figure 1 The dynamic change of CTC status at three different time points during following-up time. A total of 38 patients were CTC-positive and 34 patients were CTC-negative before chemotherapy. In all, 20 patients were CTC-positive and 52 patients were CTC-negative after three cycles of chemotherapy. There was a statistical significant difference between time points before chemotherapy and after three cycles of chemotherapy ($P<0.001$). After all cycles of chemotherapy, the number of positive patients was 18 and the number of negative patients was 54. There was no statistical significant difference between time points after three cycles and after all cycles ($P=0.7$).

Table 5 The dynamic change of CTC status in peripheral blood of operable breast cancer patients before and after chemotherapy

	CTC (postchemotherapy)		
CTC (prechemotherapy)	**Positive**	**Negative**	***P*-value**
	n (%)	*n* (%)	
Positive ($n=38$)	18 (47)	20 (53)	
Negative ($n=34$)	0 (0)	34 (100)	0.000004

Abbreviation: CTC = circulating tumour cells.

Table 4 Positive rate of the three mRNA markers in peripheral blood from different study groups

		Individual marker assay (positive rate, %)		
	Three marker assay positive rate (%)[a]	**CK19**	**hMAM**	**SBEM**
Control group				
$n=40$	2 out of 40 (5)	1 out of 40 (2.5)	1 out of 40 (2.5)	0 out of 40 (0)
Operable breast cancer				
$n=94$	53 out of 94 (56)	46 out of 94 (49)	32 out of 94 (34)	12 out of 94 (13)
P-value	0.00002	0.00003	0.0007	0.0259

Abbreviations: *CK19* = cytokeratin 19; *hMAM* = human mammaglobin; *SBEM* = small breast epithelial mucin. [a]A test result was defined positive if any of the three markers was positive, and negative otherwise.

Established clinical prognostic markers (that is, axillary lymph node metastasis, negative ER/PR status and positive HER2 status) cannot precisely predict the relapse for individual patient, but CTC are supposed to represent occult burden of systemic disease and hopefully become another predictive marker for early-stage breast cancer patients. In this study, we explored the correlation between detection of multimarker-positive CTC and clinicopathological characteristics of patients with operable breast cancer. We found that there was no significant association between detection of multimarker-positive CTC and any established clinical prognostic markers, which was consistent with other research (Xenidis *et al*, 2006; Apostolaki *et al*, 2007; Mikhitarian *et al*, 2008; Chen *et al*, 2010; Molloy *et al*, 2011). Therefore, we hypothesised that the occurrence of CTC may be independent with established prognostic markers and CTC may be another independent prognostic factor for breast cancer.

We monitored the dynamic change (before and after systemic adjuvant chemotherapy) of CTC status in peripherial blood of operable breast cancer patients. Results showed that after three cycles of chemotherapy, 47% (18 out of 38) of patients who were CTC-positive before chemotherapy changed into negative status. And after all cycles of chemotherapy were finished, another two patients changed into negative status, which means 53% (20 out of 38) of patients who were CTC-positive before chemotherapy changed into negative status. It indicates that systemic adjuvant chemotherapy could kill CTC in some patients, and this effect could be observed after three cycles of chemotherapy. Notably, 47% (18 out of 38) of patients remained CTC-positive after chemotherapy, which suggests that the chemotherapy regimen offered may be less effective or inactive for some patients. For the former patients, effective chemotherapy regimen may be needed whereas for the latter ones, the failure of adjuvant chemotherapy may be due to insensitivity of chemotherapeutic drugs offered or the presence of dormant cancer stem cells, which were insensitive to adjuvant chemotherapy (Abraham *et al*. 2005; Balic *et al*, 2006). For these patients, therefore, new therapies may be needed, as either changing chemotherapeutic drugs or using other therapies.

In conclusion, we have established a sensitive and specific platform to detect CTC in peripheral blood of breast cancer patients. We found that systemic adjuvant chemotherapy had a significant impact on CTC status, and this effect could be observed after three cycles of chemotherapy. Therefore, CTC detection had the potential to be used to evaluate the efficacy of adjuvant chemotherapy immediately after chemotherapy was finished in operable breast cancer patients. However, owing to the short follow-up time, our results should be interpreted with caution until verified from a long-term follow-up aimed to prognosis.

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